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(54) Title: METHOD OF PRODUCING A LYOPHILIZED LIPOSOME PRODUCT

(57) Abstract

A method of forming a lyophilized liposome product by combining a lipid with an aqueous solution containing a drying protectant, without adding organic solvent, agitating the mixture to form a population of liposomes, and then lyophilizing the mixture to form a lyophilized liposome product. The invention is also directed to the lyophilized products obtained by the method, and to liposomes prepared by reconstituting the lyophilized product. The lyophilization step may be carried out at higher drying temperatures resulting in, *inter alia*, a more cost effective process.

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METHOD OF PRODUCING A LYOPHILIZED LIPOSOME PRODUCT

5 The present invention is generally directed to a method of producing lyophilized liposomes and particularly to a method in which an organic solvent, typically used for dissolving the lipid and other components of the process, is eliminated. This enables the liposomes to be lyophilized in a more efficient
10 and less costly manner.

Methods of forming liposome vesicles for the association of a bioactive agent are well known. As used herein the term "association" shall mean bioactive agent which is encapsulated within the liposome and bioactive agent which, while
15 not encapsulated, remains with the liposome and is not readily separated therefrom.

Some methods of forming liposomes employ an organic solvent to dissolve a lipid alone or the lipid and a bioactive agent such as a drug. For example, in Bally et al., U.S. Patent
20 No. 5,077,056, lipids are dissolved in an organic solvent and combined with an aqueous medium to form liposomes. Then a bioactive agent such as a drug is loaded into the preformed liposomes using a transmembrane concentration gradient. On the other hand, in Lenk et al., U.S. Patent No. 5,082,664, a lipid
25 and a bioactive agent are dissolved together in an organic solvent, and combined with an aqueous medium to form liposomes associated with the bioactive agent. In particular, the lipid and the bioactive agent (e.g. lipophilic drugs such as the prostaglandins) are co-dissolved in an aqueous-miscible organic
30 solvent such as ethanol, then added slowly to an aqueous solution, which may additionally contain a drying protectant and/or a buffer, as discussed in the Lenk et al. patent. Both of these patents are hereby incorporated by reference into the present disclosure.

35 Another method for forming liposomes employs ethanol injection and is discussed in Batzri et al., Biochem. Biophys.

Acta. 298:1015 (1973). The ethanol injection method has been used to form liposomes having associated therewith a lipophilic or hydrophilic bioactive agent. When forming liposomes containing a lipophilic bioactive agent (e.g. prostaglandin), an optional preservative and the bioactive agent are added to the ethanol containing lipid. The resulting mixture is then slowly added to an aqueous medium. This process forms liposomes entrapping the aqueous medium. Ethanol injection processes, as well as other liposome formation processes, using a desalted charged lipid are disclosed in Popescu et al., U.S. Patent No. 5,154,930, incorporated by reference into the present specification. A method of controlling size distribution of resultant liposomes in an ethanol infusion process is discussed in Aitcheson et al., U.S. Patent No. 4,994,213.

For the formation of liposomes having a hydrophilic bioactive agent associated therewith (e.g. aminoglycosides, such as gentamicin), the bioactive agent is added to the aqueous phase. The lipid and ethanol are combined to form a solution which is added to the aqueous phase and the resulting mixture is processed to form liposomes. The aqueous phase may be a solution of one or more drying protectants with or without a preservative.

The liposome preparations prepared by such methods typically contain liposomes having a wide variety of particle sizes. It is often desirable to reduce the size of the larger liposomes to obtain a single-modal size distribution encompassing a desired mean particle size. The term "single-modal size distribution" as used herein shall mean that most of the liposomes have a particle size within a continuous range of particle sizes encompassing the mean particle size. The term "mean particle size" shall mean the sum of the diameters of each liposome of the population divided by the total number of liposomes.

Size reduction to obtain a single-modal size distribution can be achieved by a number of methods such as by extrusion through a filter, as described in Pieter Cullis et al., U.S. Patent No. 5,008,050, incorporated herein by reference.

A method of sizing liposomes by filtration through a 200 nm UniporeTM polycarbonate filter is discussed in Szoka, Proc. Natl. Acad. Sci. U.S.A. 75:4194-8 (1978). A size-processing method based on liposome extrusion through a series of 5 uniform straight-pore type polycarbonate membranes is described in Hunt et al., U.S. Patent No. 4,529,561.

U.S. Patent No. 4,737,323, describes a method for sizing liposomes by extrusion through an asymmetric ceramic filter. Such filters are designed for operation at relatively 10 high pressure, and can be backflushed to prevent clogging. U.S. Patent No. 4,927,637, describes a method of sizing liposomes by passing them through a polymer filter having a web-like "tortuous-path" construction.

An alternative type of filter medium is described in 15 Furneaux et al., U.S. Patent No. 4,687,551. This patent discloses a filter sheet comprising an anodic aluminum oxide film having branched pores extending from one surface of the film to the other. The film is unique in that it includes a system of larger pores extending in from one face and a system of smaller 20 pores extending in from the other face. The system of larger pores interconnects with the system of smaller pores such that the inner ends of one or more smaller pores are joined to the inner end of a larger pore and there are substantially no larger pores that terminate within the film.

25 The application of an aluminum oxide porous film to the size reduction of liposomes is disclosed in Royden M. Coe et al., U.S. Serial No. 771,267 filed on October 4, 1991.

Homogenization is another method for size reducing 30 liposomes. In a simple homogenization method, a suspension of liposomes is repeatedly pumped under high pressure through a small orifice or reaction chamber until a desired size distribution is achieved.

In such liposome-forming methods, the resulting 35 liposomes may be dehydrated or lyophilized by any method known in the art, so that the size and contents are maintained during the drying procedure and through rehydration. It has been found that

one group of drying protectants, the saccharides, when included in the liposome formulations, are especially useful at maintaining the liposome particle size after rehydration.

For the purpose of rehydration of the dehydrated or
5 lyophilized product, an aqueous solution such as distilled water with or without buffer may be added. The pH gradient may be established by adding a relatively acidic aqueous solution to the formulation. Reconstitution may proceed at a temperature of about 20° to 70°C and the solutions diluted as needed and
10 administered.

These methods of lyophilization, however, are not as efficient as desired because of the presence of residual organic solvent in the liposome product prior to lyophilization. The solvent may make it more difficult and time consuming to
15 lyophilize the product due to the need for a lower primary drying temperature. Elimination of the organic solvent may be beneficial, for example, because the product may have a higher glass transition temperature and primary drying could take place at higher temperatures.

20 SUMMARY OF THE INVENTION

The present invention is directed to a process employing no added organic solvent for the production of a lyophilized liposome product. The process comprises combining at least one lipid with an aqueous solution containing a drying
25 protectant in the absence of added organic solvent and lyophilizing the resulting mixture to form the liposome product. The liposome product may contain a bioactive agent. The temperature needed to lyophilize the final product may not be as low as previously required in systems using an organic solvent.
30 As a result, the time and cost of the lyophilization procedure may be significantly reduced over processes which employ an organic solvent.

In accordance with the present invention, there is provided a method of forming a lyophilized liposome product comprising:

- (a) without adding organic solvent, combining at least one lipid with an aqueous solution containing a drying protectant to form a mixture;
- 5 (b) agitating the mixture to form a population of liposomes; and
- (c) lyophilizing the processed mixture containing said population of liposomes to form the lyophilized liposome product.

In a particular embodiment of the present invention, a 10 bioactive agent is associated with the liposomes. The invention is also directed to the lyophilized liposome product produced by the above method, and to compositions of liposomes produced by reconstituting the lyophilized product. The invention further provides a lyophilized liposome product comprising a lipid-15 encapsulated bioactive agent having a reduced level of organic solvent, and preferably substantially absent organic solvent.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is premised, *inter alia*, on the discovery that a lyophilized liposome product can be made without 20 adding organic solvent. Processing of liposomes in accordance with the present invention eliminates the time and cost of adding the solvent, as well as removing the solvent during lyophilization.

The liposomes of the present invention are prepared 25 by adding a lipid to an aqueous solution containing a drying protectant. A particular type of lipid material for use in this invention is one which is amphipathic in character. Hydrophilic character can be imparted to the molecule through the presence of phosphato, carboxylic, sulphato, amino, sulfhydryl, nitro, and 30 other like groups. Hydrophobicity can be conferred by the inclusion of groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group. The preferred amphipathic compounds are 35 phosphoglycerides, representative examples of which include phosphatidylcholine, phosphatidylethanolamine, lysophosphatidyl-

choline, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, dimyristoylphosphatidylglycerol and diphosphatidylglycerol alone or in combination with other lipids. Synthetic saturated compounds such as dimyristoyl-
5 phosphatidylcholine, dipalmitoylphosphatidylcholine, or distearoylphosphatidylcholine or unsaturated species such as dioleoylphosphatidylcholine or dilinoleoylphosphatidylcholine might also be usable. Other compounds lacking phosphorus, such as members of the sphingolipid and glycosphingolipid families,
10 are also within the group designated as lipid.

A variety of sterols and other sterols and their water soluble derivatives have also been used to form liposomes; see specifically Janoff et al., U.S. Patent No. 4,721,612 and references referred to therein, all of which are incorporated
15 herein by reference. Various tocopherols and their water soluble derivatives have also been used to form liposomes, as disclosed in Janoff et al. U.S. Patent No. 4,861,580, incorporated herein by reference. Preferred of this group are cholesterol hemisuccinate and tocopherol hemisuccinate.

20 The drying protectants which are employed for lyophilization in accordance with the present invention are selected from saccharides such as sucrose, dextrose, maltose, mannose, galactose, raffinose, trehalose, lactose, as well as polyhydric alcohols such as mannitol, and mixtures thereof.
25 Other drying protectants which can be employed in the present process include albumin, dextrans, or polyvinyl alcohol. Maltose is particularly preferred.

The concentration of the drying protectants is generally in the range of from about 1 to 20% by weight,
30 preferably about 5 to 10% by weight, based on the weight of the aqueous phase. The polyhydric alcohol, when present and used in addition to the saccharides, is preferably provided at a concentration of up to 2% by weight, more preferably about 1% by weight, based on the weight of the aqueous phase. The preferred
35 polyhydric alcohol is mannitol.

The bioactive agents which may be encapsulated within the lipid bilayer include nucleic acids, polynucleotides,

antibacterial compounds, antiviral compounds, tumoricidal compounds, proteins, toxins, enzymes, hormones, neurotransmitters, glycoproteins, immunoglobulins, immunomodulators, dyes, radio labels, radio-opaque compounds, fluorescent compounds,

5 polysaccharides, cell receptor binding molecules, anti-inflammatories, antiglaucomic agents, mydriatic compounds, local anesthetics, and the like. Specific examples of such active agents and their incorporation into liposomes can be found in Lenk et al., U.S. Patent No. 4,522,803; Fountain et al., U.S.

10 Patent No. 4,588,578; Janoff et al., U.S. Patent No. 4,861,580 and 4,897,394; and Lenk et al., U.S. Patent No. 5,082,664; each of which is incorporated herein by reference.

The bioactive agents which find particularly effective application to the present invention are lipophilic bioactive agents, particularly arachidonic acid metabolites including their structural analogs and synthetic enzyme inhibitors. One class of such arachidonic acid metabolites is the group of bioactive agents known as prostaglandins including, but not limited to prostaglandin E₁.

20 Hydrophilic bioactive agents, such as the aminoglycoside antibiotics and their structural analogs, are examples of hydrophilic bioactive agents. These include gentamicin, streptomycin, dihydrostreptomycin, tobramycin, neomycin B, paromycin, ribostamycin, lividomycin, kanamycin, 25 viomycin, sisomicin, netilmicin and amikacin, as well as analogues and derivatives thereof. Gentamicin is the preferred aminoglycoside antibiotic.

The process of forming liposomes, in accordance with the present invention is essentially the same for lipophilic and 30 hydrophilic bioactive agents. For bioactive agent associated liposomes, an optional preservative such as disodium EDTA and the bioactive agent (e.g. prostaglandin E₁ or gentamicin) are added to an aqueous medium, preferably a solution of a drying protectant, most preferably a maltose solution at a preferred 35 concentration of about 5 to 10% by weight based on the total weight of the aqueous phase. The liposomes are prepared at a

temperature above the phase transition temperature of the lipid membrane.

The resulting bulk liposomes, whether or not the bioactive agent is associated therewith, may if desirable, 5 undergo size reduction. Size reduction may be accomplished by utilizing any one of the methods described hereinbefore to obtain a single-modal size distribution of liposomes encompassing a desired mean particle size.

The size reduction of the liposomes is preferably 10 conducted by extruding the liposomes through filters having straight through or tortuous paths, according to the procedure disclosed in U.S. Serial No. 07/771,267 filed October 4, 1991, using an AnoporeTM filter or by homogenization such as by the use of a Microfluidizer to form a single-modal size distribution, 15 preferably having a mean particle size in the range of no more than 200 nm, most preferably 150 to 190 nm.

The bulk liposomes produced by the process of the present invention may be separated from unassociated bioactive agent, if necessary, as well as from free lipid, salts and water 20 by the common technique of ultrafiltration such as disclosed in Munir Cheryan, Ultrafiltration Handbook, pp. 205-213 and 377, Technomic Publishing Company (1986).

Diafiltration is one such ultrafiltration system in which permeable solutes are removed by the addition of fresh 25 solvent or other solution to the feed liquid. The remaining liquid (the retentate) containing non-permeated substances including the desired liposome product is recovered. A preferred method of diafiltration is disclosed in Lenk, et al., PCT Published Application No. WO89/00846, the disclosure of which is 30 incorporated herein by reference.

Diafiltration systems typically employ a filter device having one or more primary pathways formed by a porous filter composition. The filter device has a rated pore size such that generally materials having a size equal to or less than the rated 35 pore size will be able to pass through the filter device via narrower secondary pathways. Generally, the larger components

will remain in the primary pathways and pass through the filter device as part of the liquid retentate. When liposomes are prepared using a diafiltration system, the liposomes pass out of the filter device through the primary pathways while the
5 permeable solutes pass through the narrower secondary pathways.

The dehydration or lyophilization of the liposomes of the present invention may be performed by any methods known in the art for dehydrating or lyophilizing liposomes. For dehydration, for example, the liposomes may be dried according to
10 the procedures of Janoff et al., U.S. Patent No. 4,880,635,
incorporated herein by reference.

The liposomes of the invention are preferably lyophilized by first pre-cooling the liposomes in a vessel at a temperature of from about 0 to 8°C and then freezing the pre-
15 cooled liposomes to a temperature of from about -50 to -38°C, preferably about -40°C. Thereafter, the pressure of the vessel is reduced while raising the shelf temperature to a temperature of from about -18 to -22°C, preferably about -20°C until the product and shelf temperature equilibrate. Once the primary
20 drying stage is completed, secondary drying is commenced by raising the shelf temperature to about 36 to 40°C, preferably about 38°C, and maintaining that temperature until the water content is reduced to below about 2% by weight, preferably to below about 1% by weight. The lyophilized liposome formulation
25 prepared in this manner in the absence of an organic solvent may be stable for at least one year when stored at temperatures of up to 25°C.

When the lyophilized liposomes are to be used, rehydration can be accomplished by adding an aqueous solution,
30 e.g., distilled water, water for injection (WFI), or buffer or aqueous solution of appropriate pH, as described above, to the liposomes, and gently agitating them to rehydrate and suspend them. The rehydration may be performed at about room temperature, that is 25°C. If the bioactive agent was
35 incorporated into the liposomes prior to dehydration, and no further composition changes are desired, the rehydrated liposomes

can be used directly in the therapy following known procedures for administering liposome associated drugs.

During preparation of the liposomes as described above, organic solvents are not used to suspend the lipids and/or 5 the active agent, such as prostaglandin or gentamicin. It being understood, however, that minor amounts of residual solvent may be present in components used to make the liposomes including the lipids and perhaps the bioactive agent. Accordingly, the final liposome product contains no residual organic solvent other 10 than very small amounts which may be present in the raw materials used to make the liposomes.

The resulting liposome product may be freeze dried at higher temperatures than liposomes containing an organic solvent such as ethanol.

15 For example, a bioactive agent such as prostaglandin can be added to an aqueous solution containing a drying protectant, such as maltose and mixed in a reactor equipped with an impeller. The lipid, such as egg phosphatidylcholine, can then be added to the reaction vessel 20 without mixing. After the addition, mixing can be commenced again to produce a liquid medium containing a heterogeneous (non-uniform) size distribution of liposomes associated with the bioactive agent. In preferred embodiments, much of the bioactive agent is encapsulated as part of the aqueous phase within the 25 liposomes.

The resulting bulk liposome medium can be extruded through a filter, such as a branched-pore aluminum oxide filter, and then sterilized by filtration to form a single-modal size distribution of liposomes.

30 The resulting liposomes can then be lyophilized, for example, as follows. The liposomes can be placed in a freeze dryer that is preferably pre-cooled to about 5°C and then frozen by lowering the shelf temperature to preferably about -42°C. Once the liposomes reached -40°C, primary drying can be initiated 35 by lowering the pressure of the vessel, preferably to about 0.150 mm Hg and raising the shelf temperature, preferably to about 20

°C, which is preferably maintained until the product and shelf temperature equilibrate. Upon completion of the primary drying cycle, secondary drying can be commenced by raising the shelf temperature, preferably to about 38°C and preferably maintaining that temperature for about 7-8 hours. According to the following example, this process can result in 99.6% liposomes having a size between 50 nm and 450 nm, and the lyophilized liposome product having a water content of 0.9%.

The liposomes resulting from the processes of the present invention can be used therapeutically in mammals, including man, in the treatment of infections or conditions which require the sustained delivery of the drug in its bioactive form. Such conditions include, but are not limited to, disease states such as those that can be treated with prostaglandins or aminoglycosides.

The process of the present invention is capable of producing a single-modal size distribution of liposomes under less severe and time consuming conditions than are possible when the liposomes are prepared using an organic solvent.

20

EXAMPLE 1

20.0 µg of prostaglandin E₁ (PGE₁) was added to 800 mL of aqueous solution containing 880 mg/mL of maltose and mixed for 10 minutes in a 3 liter Applikon™ reactor equipped with 3 baffles and a Lightnin™ R-100 impeller.

8.8 mg of egg phosphatidylcholine were added to the reaction vessel without mixing. After the addition, mixing was commenced again with the impeller rotating at the rate of 1,995 rpm for 30 minutes to produce a liquid medium containing a heterogeneous (non-uniform) size distribution of liposomes associated with the PGE₁. In particular, much of the PGE₁ is encapsulated as part of the aqueous phase within the liposomes.

The resulting bulk liposome medium was then extruded through a 100 nm backed Anopore™ branched-pore aluminum oxide filter (manufactured by Whatman Corp. of Banbury Oxon, United

Kingdom) and then sterilized by filtration using a 220 nm Millipak™ 100 filter to form a single-modal size distribution of liposomes.

The resulting liposomes were then lyophilized in the
5 following manner:

- (1) 5 mL of liposomes in a 20 mL vial were placed in an FTS™ freeze dryer and pre-cooled to 5°C;
- (2) The pre-cooled product was then frozen by lowering the shelf temperature to -42°C;
- 10 (3) Once the liposomes reached -40°C, primary drying was initiated by lowering the pressure of the vessel to 0.150 mm Hg and raising the shelf temperature to 20°C which was maintained until the product and shelf temperature equilibrated; and
- 15 (4) Upon completion of the primary drying cycle, secondary drying was commenced by raising the shelf temperature to 38°C and maintaining that temperature for 7-8 hours. An analysis of the resulting lyophilized product is shown in Table 1.

20

TABLE 1

Water Content (%)	0.9
pH	4.2
Osmolality (mosmol/kg)	302
Particle Size	Mean (nm)
	158 nm
% < 50 nm	0.2
50 nm < % < 450 nm	99.6
% > 450 nm	0.2
Total PGE ₁ (μ g/vial)	94
Free PGE ₁ %	2
Total Phospholipid (mg/vial)	44

WHAT IS CLAIMED IS:

1. A method of forming a lyophilized liposome product comprising:
 - 5 (a) without adding organic solvent, combining at least one lipid with an aqueous solution containing a drying protectant to form a mixture;
 - (b) agitating the mixture to form a population of liposomes; and
- 10 (c) lyophilizing the mixture containing said population of liposomes to form the lyophilized liposome product.
2. The method of claim 1 further comprising associating a bioactive agent with said liposomes.
3. The method of claim 2 wherein the bioactive agent is a drug.
 - 15 4. The method of claim 3 wherein the bioactive agent is an arachidonic acid metabolite.
 5. The method of claim 4 wherein the arachidonic acid metabolite is a prostaglandin.
 - 20 6. The method of claim 5 wherein the prostaglandin is prostaglandin E₁.
 7. The method of claim 3 wherein the drug is an aminoglycoside antibiotic.
 - 25 8. The method of claim 7 wherein the aminoglycoside antibiotic is gentamicin.
 9. The method of claim 1 wherein the drying protectant is selected from the group consisting of saccharides, polyhydric alcohols, albumin, dextrans and polyvinyl alcohols, and mixtures thereof.
 - 30 10. The method of claim 9 wherein the drying protectant is present in an amount of about 1 to 20% by weight based on the weight of the aqueous solution.

11. The method of claim 10 wherein the amount of the drying protectant is about 5 to 10% by weight based on the weight of the aqueous solution.

12. The method of claim 9 wherein the drying protectant is 5 a saccharide selected from the group consisting of sucrose, dextrose, maltose, trehalose and lactose.

13. The method of claim 9 wherein the drying protectant is the polyhydric alcohol mannitol.

14. The method of claim 9 wherein the drying protectant 10 comprises a mixture of a saccharide and a polyhydric alcohol, said polyhydric alcohol being present in an amount of up to 2% by weight based on the weight of the aqueous solution.

15. The method of claim 1 wherein the population of liposomes obtained from step (b) is size reduced.

16. The method of claim 15 wherein said size reduction is effected by extruding the population of liposomes obtained from step (b) of claim 1 through a filter device to reduce the size of the liposomes.

17. The method of claim 1 wherein the step of lyophilizing 20 the processed mixture comprises:

- (a) pre-cooling the processed mixture in a vessel;
- (b) freezing the pre-cooled mixture; and
- (c) heating the frozen mixture under reduced pressure to remove water and form the 25 lyophilized liposome product.

18. The method of claim 17 wherein the water content of the lyophilized liposome product is reduced to less than 2% by weight.

19. The method of claim 17 wherein the step of freezing 30 the pre-cooled mixture is conducted at a temperature of about -38 °C to about -50°C.

20. A lyophilized liposome product produced by the process of claim 1.

21. A composition comprising a population of liposomes produced by reconstituting the lyophilized liposome product of claim 20.

22. A lyophilized liposome product comprising a lipid-
5 encapsulated bioactive agent wherein organic solvent is substantially absent from said product.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	DE,A,41 24 252 (KNOLL AG) 28 January 1993	1-3, 9-12, 20-22
Y	see the whole document ---	4-8, 13-19
X	EP,A,0 560 138 (BAYER AG) 15 September 1993 see the whole document ---	1-3, 9-12, 20-22
X	EP,A,0 021 337 (F. HOFFMANN-LA ROCHE & CO) 7 January 1981 see page 5; example 1 ---	1-3, 9-12, 20-22 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

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		CA-A-	1270197	12-06-90
		CA-A-	1270198	12-06-90
		CA-A-	1283604	30-04-91
		CA-A-	1294548	21-01-92
		CA-A-	1305054	14-07-92
		DE-D-	3587639	02-12-93
		DE-T-	3587639	24-03-94
		DE-D-	3587640	02-12-93
		DE-T-	3587640	31-03-94
		EP-A-	0191824	27-08-86
		EP-A-	0190315	13-08-86
		EP-A-	0561424	22-09-93
		JP-T-	62500101	16-01-87
		JP-T-	62500102	16-01-87
		WO-A-	8601102	27-02-86
		WO-A-	8601103	27-02-86
		US-A-	5077056	31-12-91
		CA-A-	1329548	17-05-94
		US-A-	5409704	25-04-95
		US-A-	4975282	04-12-90
		US-A-	5171578	15-12-92
		US-A-	4885172	05-12-89
		US-A-	5399331	21-03-95
		US-A-	5059421	22-10-91
		US-A-	5047245	10-09-91